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EFFECT OF FERMENTATION PARAMETERS AND VEGETABLE OILS ON PRODUCTION OF GAMMA LINOLENIC ACID BY *MORTIERELLA RAMANNIANA.*

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ABSTRACT

Aim: To optimize the production of gamma linolenic acid by *Mortierella rammaniana*
Methodology and Result: Effect of carbon source, nitrogen source, initial pH and oil supplementation was studied. The production of gamma linolenic acid reached 920 m gl^{-1} when the production media were composed of 10% glucose, 1% peptone and 0.1% yeast extract pH 6.5 and incubated at 30°C for 144 hrs. Carbon and nitrogen sources showed major effect on the increase in gamma linolenic acid production. Repression in Δ 6-desaturase activity was observed in the presence of supplemented oil.
Conclusion, Significance and Impact of Study:This study highlights conditions for increasing gamma linolenic acid production by *Mortierella rammaniana* and an insight into rapidly gaining high production of polyunsaturated fatty acids.

KEYWORDS **Lipid; gamma; Linolenic acid; *Mortierella ramanniana*; Fatty acid; culture**

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INTRODUCTION

Research has shown that essential fatty acids (EFA) are integral to proper nutrition. It has been found that EFA deficiency causes a host of ailments in animals, including humans. EFAs can reverse, as well as prevent, some human disorders and diseases (Dyal *et al*. 2005). One such essential fatty acid is Gamma Linolenic acid (GLA), which is an important intermediate in the biosynthesis of biologically active prostaglandins from linoleic acid. GLA is a potent essential polyunsaturated fatty acid. It enters into the prostaglandin pathway by elongations and desaturations through the intermediate PUFA's, dihomo-gamma-linolenic acid and arachidonic acid, respectively (Mahajan and Kamat 1995). For the smooth functioning of human metabolism the content of GLA in plasma should be about 25 mg l^{-1} (James and Carter 1998). There is evidence for the therapeutic value of this acid in treatment of atopic eczema, diabetic neuropathy, rheumatoid arthritis, cirrhosis of the liver, psychiatric disorders and premenstrual breast pain, (Somashekar. *et al*. 2003). GLA possibly plays a role in the improvement of AIDS condition (Begin and Das 1986). A number of factors have been found to affect both the fatty acid compositions and the percentage of lipid bodies found in these fungi, which imply that the lipid composition of these moulds can be manipulated in order to obtain the fatty acids of interest. The task is complicated by the fact that different species demonstrate varying optimization conditions. Current interest in the nutritional role of PUFA has stimulated research into their production from a number of fungal sources. In this work we report on the effects of physico-chemical parameters on GLA production by *Mortierella ramanniana*.

MATERIALS AND METHODS

Microorganism and cultivation

Mortierella ramanniana used in this study, was obtained from culture repository of Regional Research Laboratory, India, and was maintained on potato dextrose agar slants. The culture was grown on a medium comprising of (g l^{-1}) glucose 30, peptone 10, yeast extract 1, pH 6.5 (Control Medium). Fermentation was done in 500 ml Erlenmeyer flasks containing 100 ml medium at 28 ±2ºC on a rotary shaker at 220 rpm for 144 hrs. The mycelium after fermentation was harvested by filtration through whatman no 1 filter paper, washed with distilled water and gently dried at 50ºC for 15 hrs or till the weight is nearly constant. Triplicate standards of each flask were prepared to appraise average.

Lipid extraction

The mycelium was harvested by filtration through Whatman no.1 filter paper and washed with distilled water. The biomass thus obtained was disrupted and homogenized in a pestle and mortar using acid washed sand (1:2) and then acid hydrolyzed for 45 min with 50 ml of 0.25 mol l^{-1} HCl. Lipid was extracted from the fungal biomass with chloroform: methanol (2:1) for 3 h according to method of Folch *et al* (1957). Anhydrous sodium sulphate is added to the extracted lipids in order to remove any residual moisture. The solvent was removed by evaporating on rotavapour and the total lipid estimated. All values were means of triplicate determination.

Fatty acid determination

The fatty acid profile of mycelium was determined by saponification followed by methylation for conversion of fatty acids to corresponding methyl esters. FAMES were prepared according to the methods of Christopherson and Glass (1969) and analyzed first by TLC followed by gas chromatography fitted with a FID detector.

Thin Layer Chromatography

All comparative TLC analysis were carried on Merck 0.25mm silica gel plates developed in solvents hexane/ethyl acetate 9:1 GLA Methyl ester was detected with 1% cerric ammonium sulphate reagent after gentle heating. GLA appeared as colored spots (Yokochi *et al* 1990). All values were means of triplicate determination.

Gas Liquid Chromatography

The analyses of fatty acid methyl esters were done on Gas chromatograph (NUKON 5765) using Agilent (India) fused capillary column DB-23 (30M*0.25mmID*0.25μmT) containing 70% cyanopropyl (equi) polysil phenylene. The operating conditions were; column temperature 150°C, injection temperature 230°C, and detector temperature 250°C. Column temperature was programmed to rise 5°C min^{-1} and the final temperature was 230°C. Nitrogen was used as carrier at a flow rate of 1 ml min^{-1}. Individual fatty acids were identified by comparing with the retention times of authentic fatty acid standards obtained from Sigma Co. USA. All values were means of triplicate determination.

Gas Liquid Chromatography –Mass Spectroscopy

The product was identified on DB-225 column (Agilent) with similar conditions as described above using coupled gas chromatography and mass spectroscopy. Individual fatty acids were identified by comparing retention times and mass of GLA methyl ester with authentic fatty acid standards obtained from Sigma Co. USA. All values were means of triplicate determination.

GLA production as a function of culture time

Time course studies of lipid and GLA production was done by growing the cultures in 100 ml of growth medium in 500 ml capacity Erlenmeyer flasks at 28 ±2ºC for 144 h on a rotary shaker at 220 rpm. Cultures were harvested after every 24 h and washed with 100 ml distilled water. Lipid and GLA were estimated as described earlier. All values were means of triplicate determination.

Optimization of physicochemical parameters

To see the effect of different carbon sources on growth and fatty acid content glucose in the control media was replaced by sucrose, fructose, lactose and starch. The effect of concentration used was 3%, 5% and 10% of above mentioned carbon sources. Nitrogen source in the control medium was replaced by 1% Tryptone, malt extract and yeast extract. The effect of pH was studied by adjusting the initial pH of the medium in the range of 5.5 to 9.5 and inoculum age from 24 hrs to 72 hrs was used for further optimization studies. All values were means of triplicate determination.

Effect of oil supplementation

Effect of oil amendment was studied by growing the fungal cultures on control medium replacing glucose by various vegetable oils (4%) to be used as sole carbon source. Various oils used were peanut oil, olive oil and sunflower oil. The effect of vegetable oil when used in combination with acetate was studied using 3% vegetable oil as carbon source with 1% sodium acetate supplementation (corresponding to 56.4 g of glucose as done by Valeria *et al*. 2003). Fermentation was carried out for 144 hrs at 30°C in shaking conditions, then harvested by filtration with Whatman No.1 filter paper. To remove the excess oil entrapped within the mycelium, washing was done with Tween 80 in distilled water at a concentration of 50 μl l^{-1} followed by chloroform, methanol and then again with distilled water (Kendrick and Ratledge 1996). All values were means of triplicate determination.

Scanning electron microscopy

Recovered spores were fixed and processed using the modified method of Millionig (1961). The spores were recovered on a clean coverslip and fixed in 2.5% (v/v) glutaraldehyde in 0.1 mol l^{-1} phosphate buffer (pH 7.2) for 2 h. The material was postfixed in 1% (v/v) osmium tetraoxide in the same buffer for 3 h, dehydrated in a graded ascending acetone gradient (10%-100%) and dried using carbon dioxide, the samples were then mounted on stubs, coated with carbon in a JEOL-JEE 4X vacuum evaporator, and then coated with gold in a Polaron sputter coater. Finally, the samples were observed in a JEOL-100 CXII electron microscope with ASID operating at 40 Kv. The mycelia and spores were observed under light and phase contrast microscope (Olympus Research Microscope VANOX, USA). All values were means of triplicate determination.

RESULTS

The results showed that the mycelium contained six types of fatty acids. Saturated fatty acids accounted for 39.3% of which palmitic acid is 36.8% and stearic acid is 2.5%. The remaining fatty acids were unsaturated, including palmitoleic 5.4%, oleic acid 41.5%, linoleic acid 5.0% and gamma linolenic acid 9.0% (fig 1) The scanning electron microscopic structures of *Mortierella ramanniana* are shown in fig 2. The biomass production in *Mortierella ramanniana* gradually increased with time after inoculation and the maximum biomass was found on 144 h. The dry weights of fungal growth are given in fig 3. There was decline in growth after 144 hrs. *Mortierella ramanniana* produced maximum lipid 8.717 g l^{-1} on 144 hrs. The time course of GLA production also indicated that *Mortierella ramanniana* produced a maximum GLA on 144 hrs. Production of GLA increased with the decrease in the accumulation of lipid content (fig 3). Effect of carbon source on production of GLA is presented in table 1.Dry biomass of 29.75 $gl^{-1}$of was obtained when glucose and fructose were used as carbon source, followed by starch, and sucrose and lactose was the least. Degree of unsaturation (no of double bonds in the product) was the highest when starch was used as a carbon source, but maximum GLA yields in the range of 850-920 mg l^{-1} (9.1%) was produced with glucose as carbon source having 9.14 gl^{-1} of lipid in the biomass. For nitrogen source, the total lipid production was higher in medium containing peptone. The amount of lipid produced in all other sources was quite less (fig 4).However by increasing the content of nitrogen in the media lipid accumulation decreases whereas biomass is affected if we lower down nitrogen content. The GLA content in the total lipid was not affected by changing the initial pH of the medium in acidic and alkaline range, whereas both the lipid and biomass content decreased in the alkaline range thus affecting the GLA yield. Optimum initial pH was found to be 6.5 whereas final pH was in the range of 4.0-4.4(fig 5). The inoculum size of 8% (v/v) was found to be optimum for production of GLA. Inoculum age had little effect on culture biomass. Lipid content of biomass and GLA yield dramatically declined when 48 h or 72 hrs old inoculum was used instead of 24 hrs inoculum, however inoculum age did not affect the proportion of GLA in lipid (fig 6).

Effect of Oil Supplementation

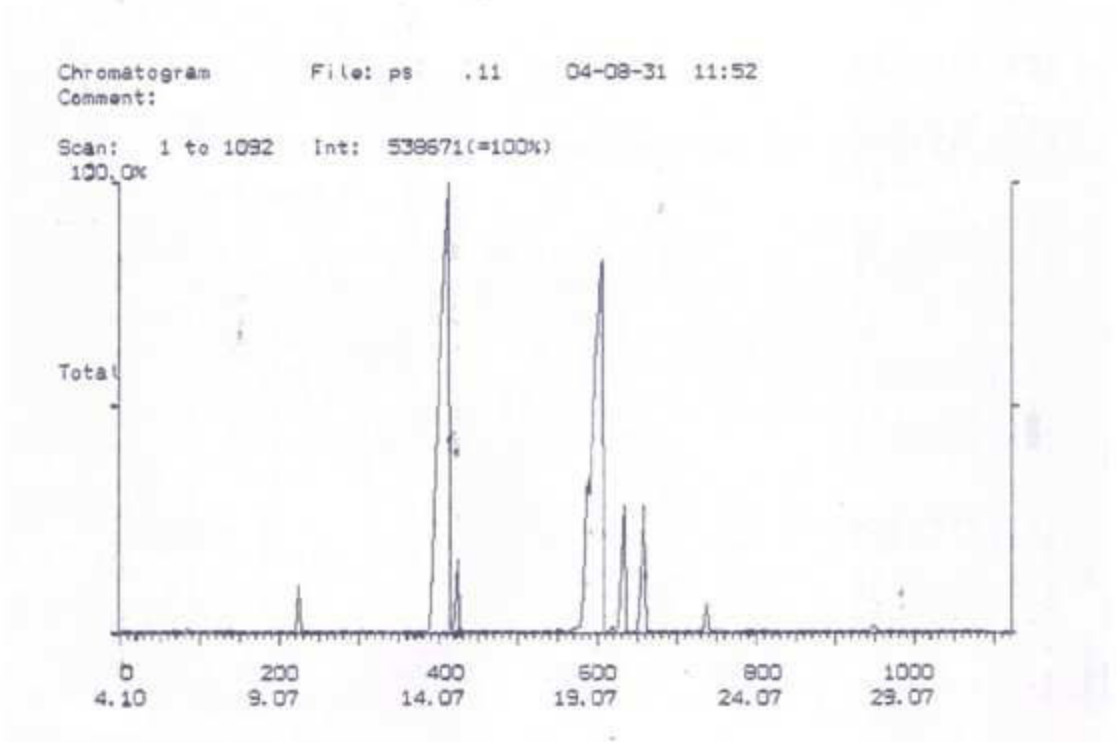
The present study shows that *Mortierella ramanniana* was able to grow in a medium supplemented with vegetable oil and sodium acetate. The culture accumulated 17-20 g l^{-1} total lipid (corresponding to 52-79% lipid in their biomass) whereas lipid content in *Mortierella ramanniana* grown on equal amount of glucose as carbon source remains 2.72 g l^{-1}. However there was no accumulation of GLA in the intracellular lipid. According to Certik 1997 formation of GLA by the same strain assimilating glucose as the sole carbon source varied from 9.1- 11.3 %. GC-MS of original commodity oil showed absence of GLA in it.

DISCUSSION

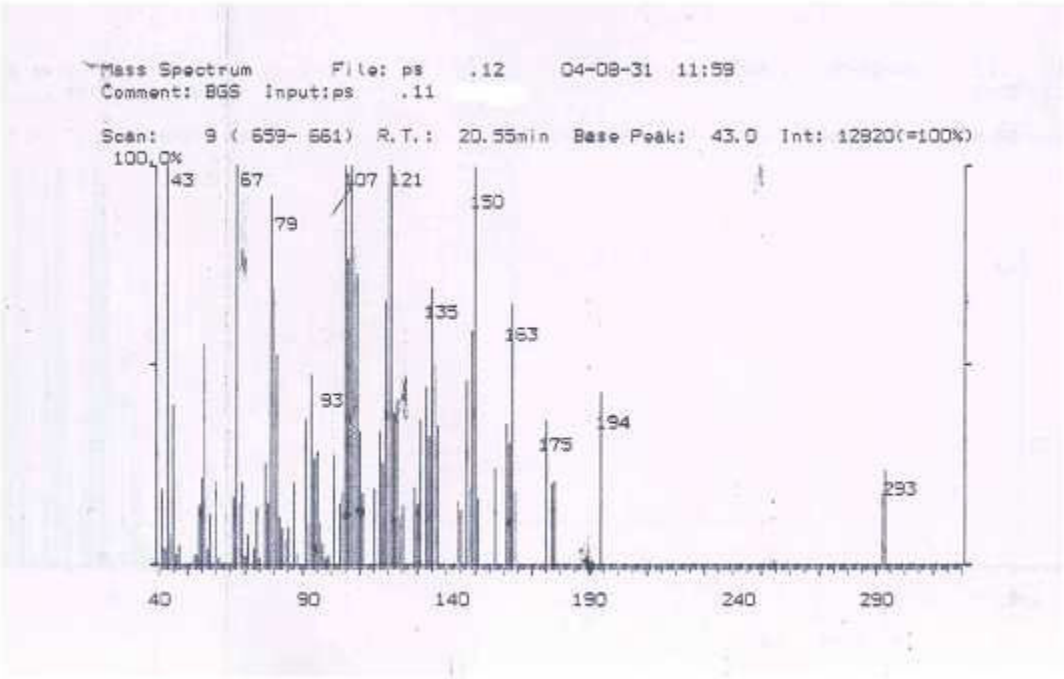
Above results and literature cited indicate that there are considerable differences in growth conditions and their interactions on the biomass and lipid yields of different fungal strain therefore it is needed to optimize growth conditions for each fungal strains. Lipid accumulation in an oleaginous microorganism begins when it exhausts a nutrient from the medium; usually this being nitrogen, but with a surfeit of carbon, usually in the form of glucose still remaining. Glucose continues to be assimilated by the cells and is converted into triacylglycerol at more or less the same rate at which lipid was synthesized during the balance phase of growth (Ratledge and Wynn 2002). However, the limitation in the supply of nitrogen means that cell proliferation is prevented, the lipid that is now formed has to be stored within the existing cells, which can no longer divide; resulting in obese microorganisms (Certik and Shimizu 1999). Keeping this in view the influence of various carbon and nitrogen sources on biomass, lipid and GLA production were studied

in this fungal culture and results are presented. Although it is generally true that oleaginous fungi accumulate lipids when their nitrogen source has become limiting (Murphy 1991), there appears to be an optimum carbon concentration at which this accumulation is maximized**.** Results of our experiments indicate that the best carbon source used is 10% glucose and 1% peptone with C: N ratio of 24:1. Further the relative insensitivity of the medium pH allows cutting down the cost of the process.

The nature of the oil added into the medium plays an important role in its utilization by microorganisms. The effect of oil supplementation into the medium on GLA and lipid production was studied by incorporating various oils and also sodium acetate along with oils. The objective was to study whether uptake of this oil as carbon source and conversion to GLA by the culture happens or not as reported earlier (Koritala *et al*. 1987; Shimizu.*et al*. 1989; Ratledge 1992; Arjuna 2013). The use of oils rich in linolenic acid should stimulate production of n6 fatty acids where the first step of linolenic acid conversion is catalyzed by delta 6 desaturase to yield GLA. Nevertheless the present study indicates that fungi accumulated LA in abundance. The biomass increased to 33.6 g l^{-1} and lipid to 20.04 g l^{-1} when olive oil plus sodium acetate was used as a carbon source,whereas 12.19 g l^{-1} and 2.72 g l^{-1} of biomass and lipid respectively with glucose grown cells (table 2) Jeffery *et al*. (1997) have also reported 3 fold increase in oil and doubling of the biomass. As fungi produced more lipids it may be thought that some of this may have been original substrate oil strongly adsorbed to the surface layers. This is unlikely as the cells were washed thoroughly with Tween 80 and distilled water, then with chloroform and methanol and finally again with distilled water. However there was cessation in the PUFA production by fungal cultures grown on vegetable oils and sodium acetate. This might be due to repression of delta 6 desaturase responsible for the conversion of linolenic acid to gamma linolenic acid, this result corroborates with that of cessation of PUFA formation in four selected filamentous fungi grown on plant oils by Kendrick and Ratledge (1996).



(a)



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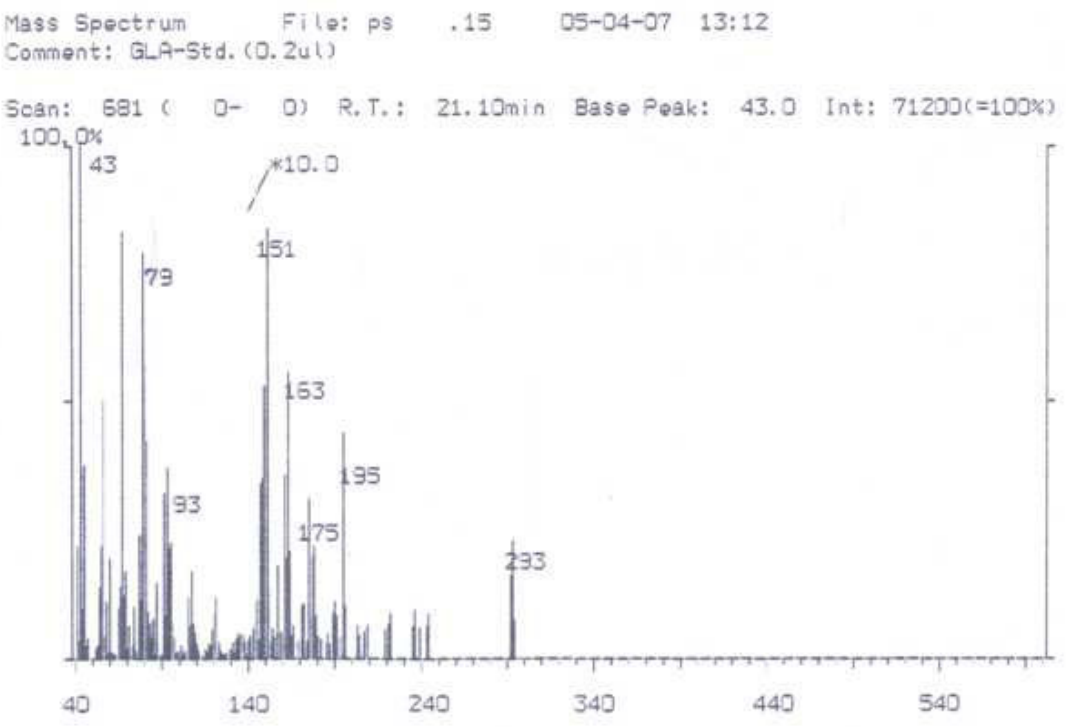

(c)

Figure 1 Evidence of Gamma linolenic Acid production by *M.rammaniana* strain based on gas chromatography and mass spectroscopy (a) Gas Chromatography of suspected GLA (b) Mass spectroscopy of suspected GLA (c) Mass spectroscopy of standard GLA.

Figure 2(a) Mycelia from submerged Cultures of M.rammaniana *after 6 days of cultivation at 400x*

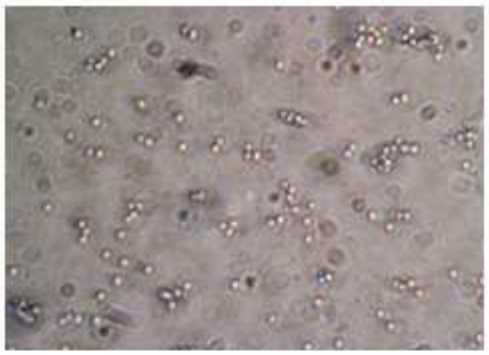

Figure 2(b) Phase Contrast microscopic view of spores of *M.rammaniana*

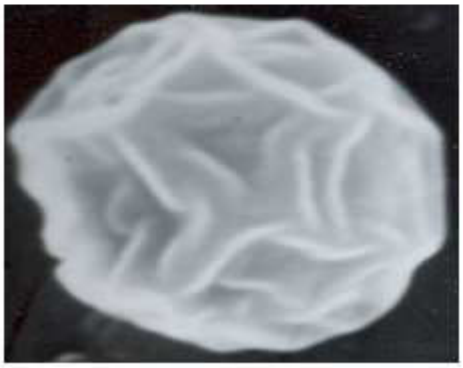

Figure 2(c) Electron Microscopic view of single spore of *M.rammaniana*

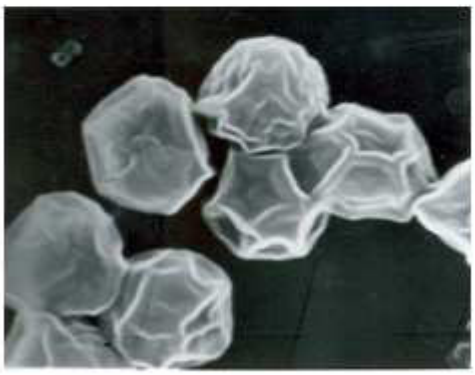

Figure 2(d) Electron Microscopic view of group of spore of *M.rammaniana*

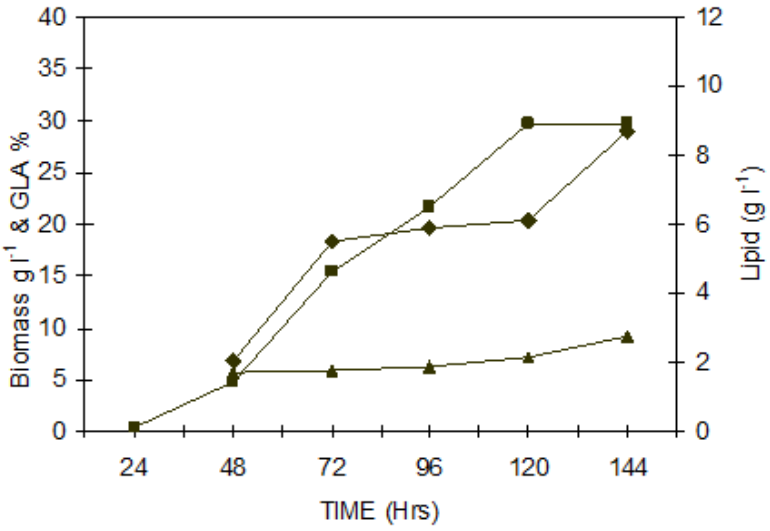

Figure 3 Time course studies of biomass, lipid and GLA production by ***M.ramanniana.*** Biomass (■); Lipid (◆);GLA (▲)

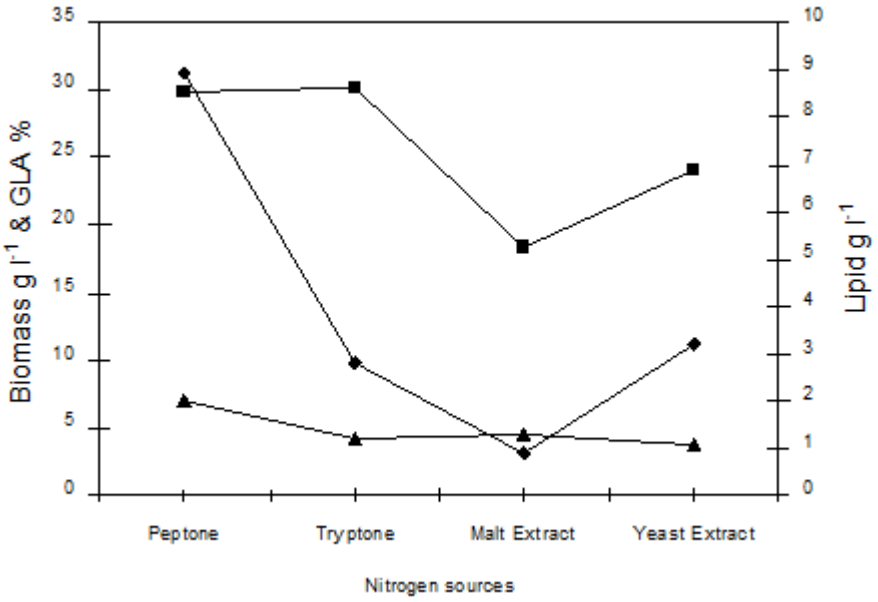

Figure 4 Influence of nitrogen sources on growth , lipid and GLA production of ***M.ramanniana***. Biomass (■); Lipid (◆);GLA (▲)

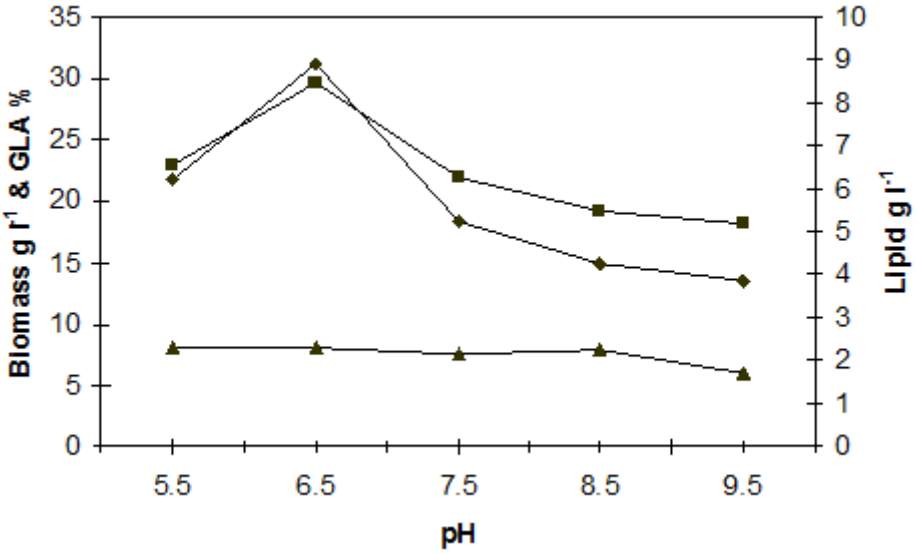

Figure 5 Influence of pH on biomass, lipid and GLA production by ***M. ramanniana*** . Biomass (■); Lipid (◆);GLA (▲)

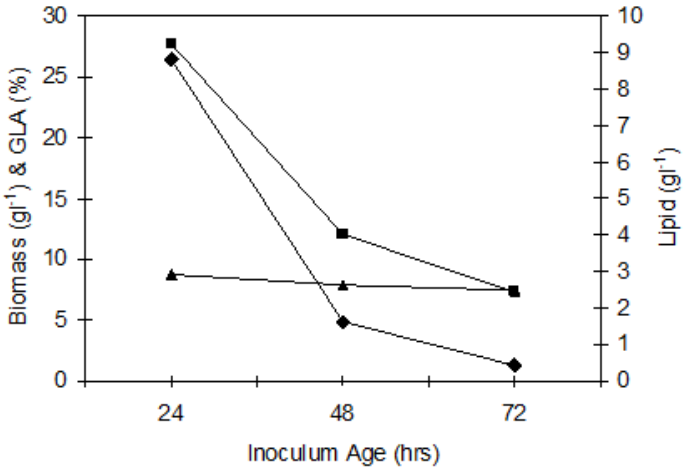

Figure 6 Influence of inoculum age on biomass, lipid and GLA production by ***M. ramanniana*** . Biomass (■); Lipid ◆);GLA ▲)

Table 1
Effect of Different Concentration of Carbon Sources on GLA production

	3%			5%			10%		
Carbon Sources	**Biomass g l^{-1}**	**Lipid g l^{-1}**	**GLA %**	**Biomass g l^{-1}**	**Lipid g l^{-1}**	**GLA %**	**Biomass g l^{-1}**	**Lipid g l^{-1}**	**GLA %**
Fructose	18.17	2.01	7.7	18.25	6.00	3.6	24.63	9.39	4.2
Glucose	15.93	1.13	11.3	12.19	2.72	8.6	29.75	9.14	9.1
Sucrose	15.26	1.53	8.7	19.53	5.53	5.0	20.20	4.02	1.5
Lactose	12.17	0.66	6.4	8.09	0.91	5.0	11.21	1.9	3.9
Starch	11.08	0.73	18.5	8.83	1.17	14.3	14.47	3.43	9.6

Table 2
Effect of oil supplementation on biomass and lipid production.

S.no	Vegetable Oil	Biomass (g/l)	Lipid (g/l)
1.	Peanut oil	7.65	3.33
2.	Peanut oil + Sodium acetate	24.9	19.7
3.	Olive oil	8.59	3.21
4.	Olive oil + Sodium acetate	33.6	20.04
5.	Sunflower oil	7.02	1.2
6.	Sunflower oil + Sodium acetate	33.34	17.63

CONCLUSION

As it is observed that the GLA content in the lipid nearly remains constant, it is desired to optimize fermentation parameters in order to maximize the biomass and lipid content, thereby finally increasing the GLA yield. Under these specific conditions, the GLA yield is at least comparable to that of evening primrose and as such the industrial GLA production in this particular fungal strain offers a viable alternative.

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